

# A Response Unit in the First Exon of the $\beta$ -Amyloid Precursor Protein Gene Containing Thyroid Hormone Receptor and Sp1 Binding Sites Mediates Negative Regulation by 3,5,3'-Triiodothyronine

ANA VILLA, JORGE SANTIAGO, BORJA BELANDIA, AND ANGEL PASCUAL

*Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, 28029 Madrid, Spain*

Thyroid hormones repress expression of APP ( $\beta$ -amyloid precursor protein) in cultured cells of neuronal origin. The effect involves binding to the nuclear thyroid hormone receptor (TR) and is mediated by DNA sequences located within the first exon of the gene. These sequences contain a thyroid hormone response element that is necessary, but not sufficient, to mediate the inhibitory effect of the thyroid hormone  $T_3$ . In this report, we show that repression by  $T_3$  is mediated by a response unit composed by the thyroid hormone response element and 5'-flank-

ing sequences that bind Sp1 and mediate stimulation by this transcription factor. In that unit, binding sites for TR and Sp1 overlap and a complex mechanism appears to account for the TR-mediated regulation of APP. Unliganded TR does not bind to DNA and allows Sp1 to bind to DNA and stimulate APP basal expression. Binding of ligand  $T_3$ , which increases affinity of TR by DNA, precludes binding of Sp1 to DNA and decreases the Sp1-dependent expression of APP. (*Molecular Endocrinology* 18: 863–873, 2004)

**A**LZHEIMER'S DISEASE IS a degenerative disorder of the central nervous system, which causes mental deterioration and progressive dementia, and is accompanied by neuropathologic lesions including the presence of senile plaques of which the  $\beta$ -amyloid protein, a hydrophobic 39- to 43-residue amino acid peptide, is the major component (for a review see Ref. 1). The  $\beta$ -amyloid protein is proteolytically derived from a set of alternatively spliced  $\beta$ -amyloid precursor proteins (APPs), which, at physiological levels, appear to be involved in neurotrophic events (2). In contrast, its overexpression might cause neuronal degeneration by a mechanism that likely involves an increased production of  $\beta$ -amyloid protein (2) and neurotoxicity (3). APP is ubiquitously expressed in mammalian tissues, and its expression can be modulated by a variety of compounds, among others the thyroid hormones. Data from our laboratory have demonstrated that  $T_3$  affects not only the splicing and secretion of APP isoforms (4), but also represses the expression of the APP gene in neuroblastoma cells, by a mechanism that requires binding of the nuclear  $T_3$  receptor (TR) to a specific sequence located in the first exon of the

gene (5). The unliganded receptor increases promoter activity and  $T_3$  reverses that activity to basal levels. Gel-mobility shift assays, using *in vitro* synthesized nuclear receptors and nuclear extracts, led to the identification of a potential negative thyroid hormone response element (nTRE), 5'-GGGCAGAGCAAG-GACG-3', between nucleotides +81/+96, which preferentially binds heterodimers of TR with the retinoid X receptor (RXR). Insertion of sequences (+55 to +102) containing this element conferred negative regulation by  $T_3$  to a heterologous tk (thymidine kinase) promoter, thus indicating its functionality.

Several mechanisms have been proposed to explain the TR-mediated transcriptional control of negatively regulated genes. In many cases, repression involves binding of TR to negative regulatory elements, which in general are located close to, and often downstream of, the transcriptional start site of the gene (6–10). TRs can bind to these sequences as monomers, homodimers, or heterodimers with RXR (6, 11, 12). However, up to now, a consensus sequence for nTREs has not yet been established, and the precise mechanisms involved in the  $T_3$ -induced repression remain unclear. In these genes unliganded TR stimulates promoter activity, and the addition of  $T_3$  causes a significant transcriptional repression. According to previous reports (13), corepressors (CoRs) could play a central role in this nTRE-mediated mechanism. In negative regulated genes, TR retains the fundamental features of interactions with CoRs, but the functional consequences of these interactions are reversed in comparison with positively regulated genes. It has been suggested that unliganded TR recruits CoRs and with-

Abbreviations: APP,  $\beta$ -Amyloid precursor protein; CAT, chloramphenicol acetyl transferase; CoR, corepressor; CRE, CREB-response element; CREB, cAMP response element-binding protein; HDAC, histone deacetylase; nTRE, negative TRE; RXR, retinoid X receptor; tk, thymidine kinase; TR, thyroid hormone receptor; TRE, thyroid hormone response element; UAS, upstream activation sequence.

**Molecular Endocrinology** is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

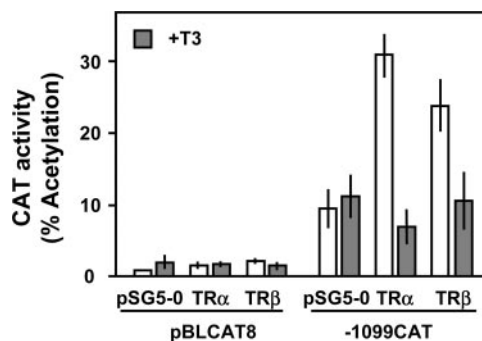
draws histone deacetylases (HDACs) from the basal promoter to cause activation (14). Upon binding of T<sub>3</sub> to TR, the CoRs are released and the induced activation reversed. In addition, other mechanisms that involve competition of TR for transcriptional cofactors such as cAMP response element binding protein (CREB)-binding protein, competition with other transcription factor-binding sites, or interaction of TR with other transcription factors such as Sp1, activator protein 1, nuclear factor- $\kappa$ B, or v-erbA-related-2, have been also proposed to explain the transcriptional control of genes by nuclear receptors (15). Moreover, it has been also described that unliganded TR can activate transcription from a number of promoters, several of which lack a TRE, thus suggesting that constitutive activity mediated by TR in the absence of ligand not always requires the presence of specific TREs and binding to DNA (8, 13).

In this report we describe a TR binding site-containing sequence in the first exon of the APP gene that mediates transcriptional inhibition by T<sub>3</sub> in N2a neuroblastoma cells. However, whereas a fragment spanning nucleotides +55/+101 was able to mediate this response, the receptor binding site located between positions +81 and +96 was unable by itself to mediate any response in the absence of the 5'-flanking sequences. Therefore, in terms of functionality, the binding site element would not be considered as a suitable responsive element. Moreover, unliganded TR can stimulate the APP promoter activity without binding to DNA. These results strongly suggest a dual mechanism in which unbound TR allows the transcriptional activity of the gene and blocks transcription when bound to the TR binding site, very likely by interfering with the response induced by transcription factor/s that recognizes the upstream and overlapping +64/+83 sequences.

## RESULTS

### Negative Regulation of APP Promoter Activity by T<sub>3</sub> Is Equally Mediated by Both the $\alpha$ - and $\beta$ -Isoforms of TR in N2a Cells

We have previously demonstrated that T<sub>3</sub> represses APP promoter activity in N2a $\beta$  neuroblastoma cells, a subclone of N2a cells that constitutively expresses high levels of TR $\beta$ . To analyze whether the effect is equally mediated by the isoform TR $\alpha$ , N2a parental cells were transiently cotransfected with an APP promoter-chloramphenicol acetyl transferase (CAT) construct and a vector expressing the  $\alpha$  or  $\beta$  TR isoforms, and then incubated for 48 h in the presence or in the absence of 200 nM T<sub>3</sub>. As shown in Fig. 1 the effect was equally observed in TR $\alpha$ - or TR $\beta$ -expressing N2a cells. In both cases, CAT activity was induced by the unliganded receptor, and T<sub>3</sub> effectively reversed the stimulation.

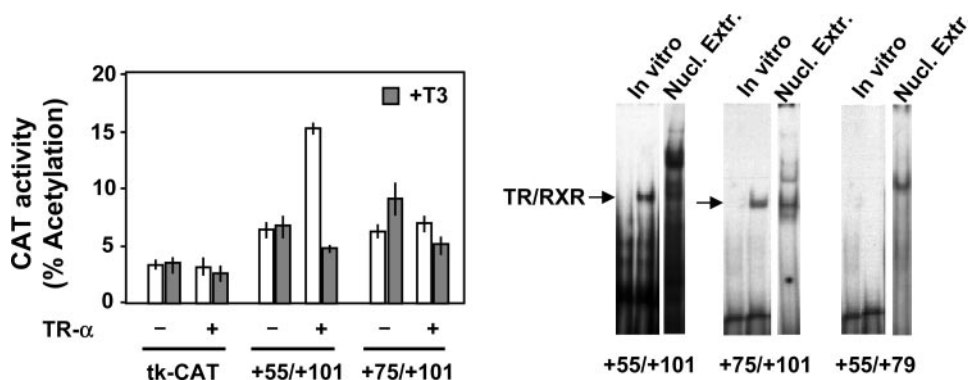


**Fig. 1.** Negative Regulation of APP Promoter Activity by T<sub>3</sub> Is Equally Mediated by Both the  $\alpha$ - and  $\beta$ -Isoforms of TR in N2a Cells

CAT activity was determined in N2a cells transiently cotransfected with a pBL-CAT plasmid containing, or not, the -1099 to +106 bp fragment of the APP promoter, and expression vectors for the  $\alpha$ - and  $\beta$ -isoforms of TR (or the empty noncoding vector pSG5-0). CAT activity was measured after a 48-h period of incubation in the absence or presence of 200 nM T<sub>3</sub>. Data are the mean  $\pm$  SD activities obtained from three independent experiments.

### The TR Binding Site Located at Nucleotide Position +81/+96 Is Unable to Transmit by Itself the T<sub>3</sub>-Induced Inhibition of the APP Promoter Activity

We have previously reported (5) that insertion of the +55/+101 sequence of APP gene, which contains the TR binding site, confers negative regulation by T<sub>3</sub> to a heterologous tk promoter, thus suggesting the functionality of the element. However, as illustrated in Fig. 2, a further 5'-flanking deletion of this fragment demonstrates that a sequence +75/+101, which contains the TR binding site, is unable to mediate repression by T<sub>3</sub> (*left panel*). N2a cells were cotransfected with a vector expressing the  $\alpha$ -isoform of the TR, and a chimeric plasmid containing either the +55 to +101 or the +75 to +101 bp fragment of the human APP gene linked to the tk-CAT reporter gene, and then treated for an additional 48-h time period with or without 200 nM T<sub>3</sub>. As shown in *left panel*, unliganded TR increased basal promoter activity, and T<sub>3</sub> reversed this effect in cells expressing the +55/+101-containing construct, but not in cells transfected with the +75/+101 plasmid. These results show that sequences contained in the deleted +55 to +74 bp fragment of the APP gene are involved in repression by T<sub>3</sub>. To determine whether deletion of this region could affect the affinity of TR by DNA, we conducted gel mobility shift assays using both *in vitro* translated receptors and nuclear extracts obtained from N2a $\beta$  cells. Results obtained are illustrated in *right panel* of Fig. 2. Bands that are compatible with mobility complexes containing the TR/RXR heterodimer were always observed when radiolabeled oligonucleotides +55/+101 and +75/+101 were used. In contrast, a sequence (+55/+79) containing the deleted fragment was unable to bind the het-



**Fig. 2.** The TR Binding Site Alone Is Unable to Mediate Repression of APP Promoter Activity by T<sub>3</sub>

*Left panel*, CAT activity in N2a cells cotransfected with a vector expressing the  $\alpha$ -isoform of the TR and chimeric plasmids containing either the +55 to +101 or the +75 to +101 bp fragment of the human APP gene. CAT activities were determined after a 48-h period of incubation with or without 200 nM T<sub>3</sub> and expressed as mean  $\pm$  SD of data obtained from duplicates obtained in two separate experiments. *Right panel*, Gel mobility shift assays using radiolabeled probes containing the +55/+101, +75/+101, or +55/+79 fragments of the APP gene and *in vitro* translated TR and RXR or nuclear extracts obtained from N2a $\beta$  cells. The mobility of the TR/RXR heterodimer is indicated by an arrow.

erodimer when either *in vitro* translated receptors or nuclear extracts were used.

#### The +75/+101 bp Fragment of the APP Gene Contains a Low-Affinity Binding Site for TR

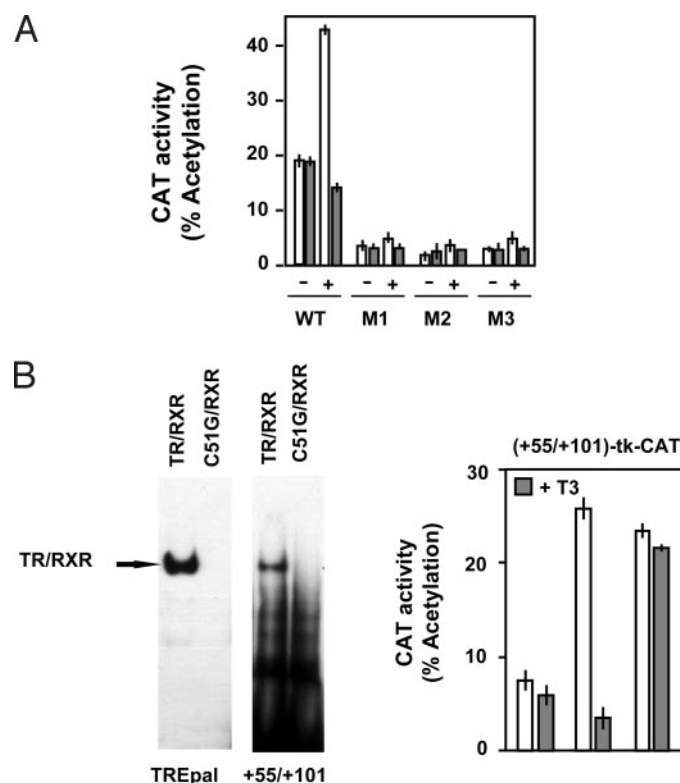
Several mechanisms have been proposed to explain the hormone-dependent and -independent effects induced by TR on negatively regulated genes. Stimulation by unliganded receptor and ligand-dependent repression generally require binding to hormone-responsive sequences located in the proximal promoter regions. However, promoter activity can be similarly regulated in other genes lacking a defined TRE (8, 13), thus suggesting that negative regulation does not require binding of TR to DNA. To prove whether the effects of unliganded and liganded TR on the APP gene require binding to DNA, we first analyzed the effects of several mutations on the TRE on regulation of the reporter plasmid by TR. As shown in panel A of Fig. 3, the reporter activity was drastically diminished by any of the mutations, and under these conditions, expression of unliganded TR and incubation with T<sub>3</sub> had little, if any, effect. This suggests that the TRE is contained in sequences that likely play a key role in basal transcription.

To further analyze the requirement for TR binding to a nTRE on the observed regulation, we also examined the effects mediated by a mutant TR (C51G), in which cysteine 51 has been changed to glycine. As illustrated in panel B of Fig. 3, this substitution eliminates binding of TR to DNA. Heterodimers of wild-type TR with RXR strongly bound to an oligonucleotide containing a consensus palindromic TRE and with less intensity to the +55/+101 fragment of the APP gene, whereas incubation with heterodimers of the C51G mutant did not result in the formation of stable DNA-protein complexes. However, as shown in *right side* of

panel B, T<sub>3</sub>-independent stimulation was equally induced by both the wild-type and the mutated receptor, indicating that ligand-independent stimulation of the APP gene promoter by TR does not require binding to DNA. In contrast, the ligand-dependent repression was mediated by the wild-type TR, but not by the C51G mutant, hence proving that ligand-dependent inhibition requires binding to specific sequences located in the APP promoter. These results suggest a mechanism that is not easily compatible with a high-affinity TR binding site. To further analyze whether in the absence of T<sub>3</sub> the receptor binds the APP promoter with low or high affinity, we examined the effects induced by a VP16-TR construct, a constitutively active receptor previously described by Tagami *et al.* (13). Results obtained after cotransfection with vectors expressing different CAT reporter genes and a vector for the VP16-TR construct are shown in Fig. 4. As expected, VP16-TR significantly stimulates the expression of a TREpal containing reporter gene. In contrast, VP16-TR did not stimulate the expression of constructs containing the +55/+101 or the -15/101 fragments of the APP gene. As a control, the reporter UAS (upstream activation sequence), which contains Gal4-binding sites and lacks a TRE, was not activated by VP16-TR. As postulated by Tagami *et al.* (13), these results strongly suggest that the T<sub>3</sub>-responsive region of the APP promoter contains a low-affinity TR binding site.

#### The Responsive Region of the APP Gene Contains Functional Sp1 Binding Sites

Computer-assisted studies of the +55/+101 region of the APP gene reveal the existence of three potential overlapping Sp1 binding sites (Fig. 5A). Sp1 is a ubiquitous transcription factor that interacts with many constitutive promoters (16) and very likely plays a role



**Fig. 3.** Stimulation of APP Promoter Activity by Unliganded TR Does Not Require Binding to DNA

A, N2a cells were cotransfected with an expression vector for TR $\alpha$  and chimeric plasmids containing the native +55/+101 fragment of the APP gene (WT) or mutants affecting the upstream hemisite (M1), the intervening sequences (M2), and the downstream hemisite (M3) of the putative TRE. CAT activity was determined after 48 h of incubation in the presence and absence of 200 nM T<sub>3</sub> and is expressed as the mean  $\pm$  SD of values obtained from two independent experiments performed in duplicate. B (*left panel*), Gel retardation assays carried out with radiolabeled probes containing the consensus element TREpal (21 nucleotides), or the +55/+101 (47 nucleotides) fragment of the APP promoter, and RXR together with wild-type or mutated (C51G) TR. Although the different size of the probes leads to different backgrounds, all lanes were run in the same gel and submitted to the same exposure time. The mobility of TR/RXR is indicated by an arrow. B (*right panel*), CAT activity determined in N2a cells cotransfected with a tk-CAT reporter gene containing +55 to +101 bp fragment of the APP promoter, and expression vectors for wild-type TR $\alpha$ , or the mutated receptor C51G. CAT activity was measured after a 48-h period of incubation in the absence or presence of 200 nM T<sub>3</sub>. Data are the mean  $\pm$  SD obtained from three independent experiments, performed in duplicate.

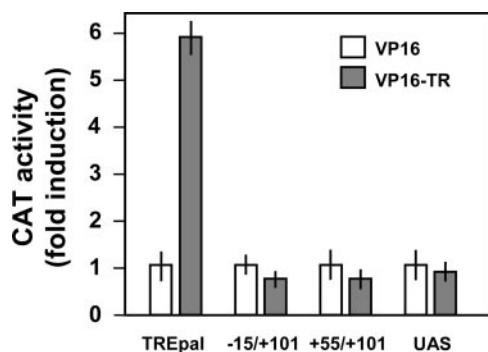
in the basal activity of the APP promoter. Moreover, because one of these putative Sp1 binding sites overlaps with the TRE identified within this region, the effects induced by T<sub>3</sub> could be the result of a direct competition between Sp1 and TR for binding to DNA. To prove whether those elements of the APP gene are able to bind Sp1, we first conducted gel mobility shift assays with purified recombinant Sp1, and <sup>32</sup>P-labeled double-stranded DNA oligonucleotides corresponding to sequences +55/+101, +75/+101, and +55/+79 of the APP gene. As shown in Fig. 5B (*left panel*), Sp1 specifically binds to the +55/+101 region. The low mobility of the retarded band could represent occupancy by multiple Sp1 proteins. However, Sp1 was unable to bind the other two analyzed sequences, showing that the complete +64/+83 region is required for binding of this factor. In addition, incubation of the +55/+101 oligonucleotide with nuclear extracts isolated from N2a $\beta$  cells led to the formation of distinct protein-DNA complexes that were further analyzed to

determine whether they contain Sp1. Results are illustrated in Fig. 5B (*right panel*). The specificity of a band, which is marked by an arrow in the graph, was demonstrated by the ability of an excess of the same unlabeled oligonucleotide (lane 2), or an unlabeled Sp1 consensus oligonucleotide (lane 3), to compete with the probe for binding to the nuclear protein. As a control, a nonrelated sequence (lane 4) was unable to affect binding. Finally, the functional importance of the Sp1 binding sites for the activity of the APP promoter was further analyzed in N2a cells cotransfected with the plasmid containing the +55 to +101 bp fragment of the human APP gene linked to the tk-CAT reporter gene, and an expression vector for Sp1. As illustrated in panel C of Fig. 5, the transient expression of Sp1 significantly stimulates CAT activity, thus providing evidence that the Sp1 elements are functional.

In addition to Sp1 sites, a putative imperfect CRE (CREB response element) was also detected in the responsive +55/+101 fragment, located between the



nucleotide positions +56 and +67. However, in contrast to the Sp1 elements, this potential CRE does not appear to participate in the regulation of APP expression because activation of CREB by incubation of cells with agents that activate adenylate cyclase was incapable of increasing expression of the CAT reporter



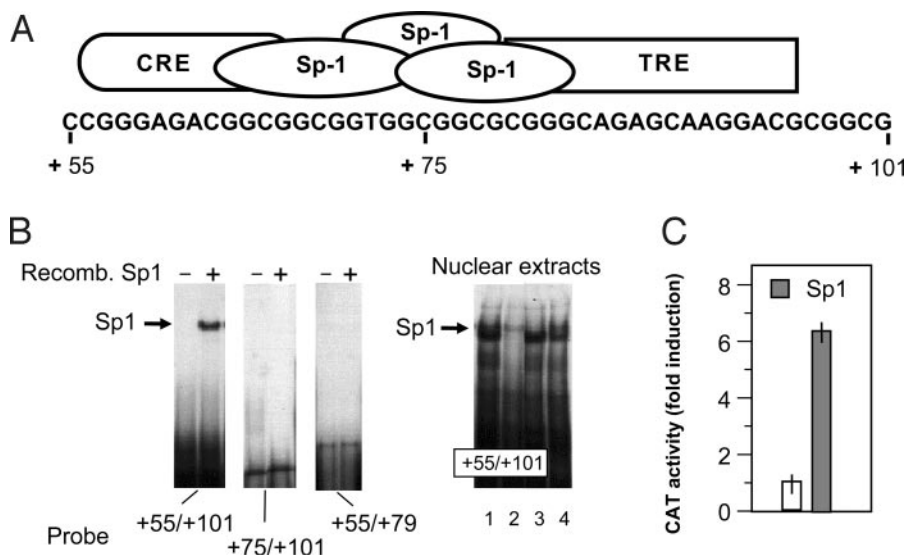
**Fig. 4.** The Responsive Region of the APP Gene Contains a Low-Affinity Binding Site for TR

VP16 or VP16-TR expression vectors were cotransfected, in the absence of T<sub>3</sub>, together with CAT reporter genes containing the consensus TREpal, or the +55/+101 and -15/+101 APP fragments. A plasmid containing the UAS-tk-CAT, which contains Gal4-binding sites (UAS) and lacks a TRE, was used as a negative control. Data, expressed as fold induction, are the mean  $\pm$  SD of CAT activities obtained from duplicates of two separate experiments.

gene (data not shown). A possible role of this element in the T<sub>3</sub>-induced inhibition of APP promoter activity was further discarded by the results illustrated in Fig. 6. N2a cells cotransfected with reporter plasmids containing the APP promoter fragments shown in panel A of the figure, together with expression vectors coding for TR (or the corresponding noncoding vector), were incubated for 48 h in the presence or in the absence of T<sub>3</sub>. As observed in panel B of the figure, the negative effect of T<sub>3</sub> was CRE independent and was equally observed in the (+55/+101)- and (+63/+101)-containing constructs. In contrast, removal of the Sp1-containing +63/+74 fragment completely abolished the inhibitory effect induced by the hormone, as previously shown in Fig. 2. These results not only discard a role of CREB, but also support a role for Sp1 in the inhibitory effect of T<sub>3</sub> on APP gene expression.

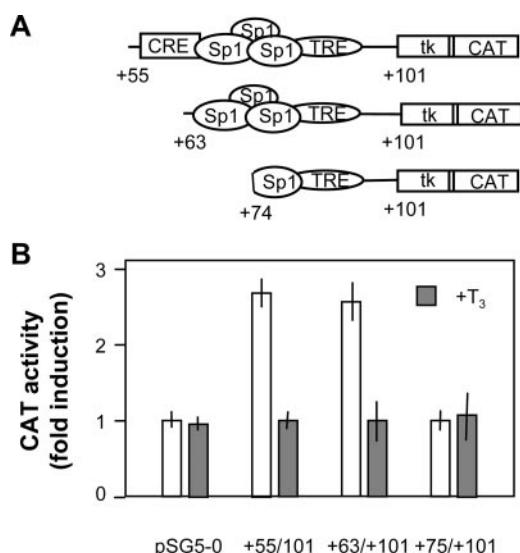
#### Competition between Sp1 and TR/RXR for Binding to the T<sub>3</sub>-Responsive Fragment of the APP Promoter

According to our results the only presence of the TR-binding site located at positions +81/+96 is not sufficient to mediate the inhibitory response of T<sub>3</sub> on the APP gene promoter. Moreover, the presence of a 5'-flanking sequence that contains binding sites for Sp1 appears to be an indispensable requirement. This suggests the existence of a T<sub>3</sub>-responsive unit that con-



**Fig. 5.** The TR-Responsive Region of the APP Gene Contains Functional Sp1 Binding Sites

A, Diagram showing the nucleotide sequence of the +55 to +101 region of the APP promoter and potential binding sites for different transcription factors. B, In the left panels, gel mobility shift assays were carried out with radiolabeled probes containing the +55/+101, +75/+101, or +55/+79 fragments of the APP gene, and recombinant Sp1. In the right panel, the radiolabeled +55/+101 was incubated with nuclear extracts, obtained from N2a $\beta$  cells, alone (lane 1) or in the presence of an excess of the unlabeled +55 to +101 oligonucleotide (lane 2), a Sp1 consensus oligonucleotide (lane 3), or a control nonrelated sequence (lane 4). The mobility of the Sp1-containing complexes is indicated by an arrow. C, CAT activity determined in N2a cells transfected with the tk-CAT reporter gene containing the +55 to +101 bp fragment of the APP promoter, and expression vector for Sp1. CAT activity was determined 48 h after transfection and is expressed as fold induction. Data are the mean  $\pm$  SD of CAT activities obtained in two independent experiments.

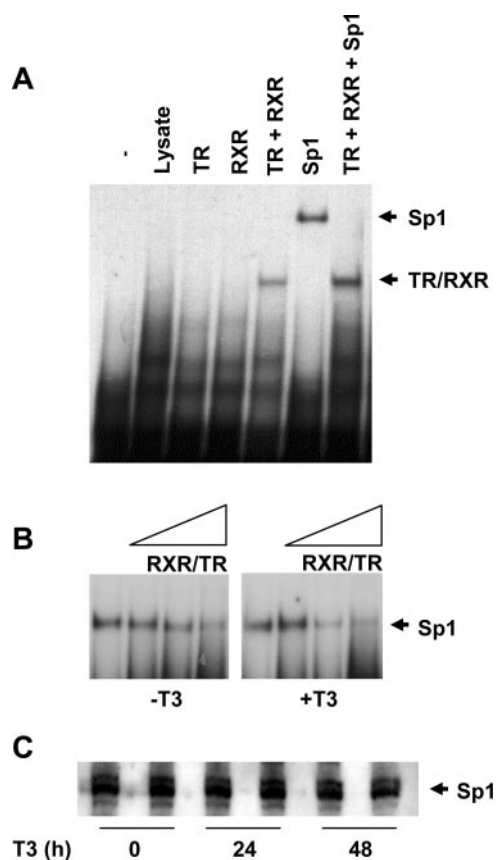


**Fig. 6.** A CRE Located at Position +56/+67 Is Dispensable for the T<sub>3</sub>-Induced Repression of APP

CAT activity in N2a cells transfected with reporter plasmids including the fragments of APP gene indicated in panel A, together with a TR $\alpha$ -expressing vector. CAT activity was determined after a 48-h period of incubation in the absence or presence of 200 nM T<sub>3</sub>. Data are the mean  $\pm$  SD of CAT activities obtained from three independent experiments and represent fold induction over basal values obtained in cells expressing a noncoding expression vector in the absence of T<sub>3</sub>.

tains not only the characteristic TRE but also Sp1 motifs. Because, as previously mentioned, binding sites for Sp1 and TR overlap in the fragment +55/+101 of the APP gene, a possible competition of both proteins for binding to the APP gene promoter could occur. To test this hypothesis, we conducted gel mobility shift assays with the TR and RXR receptors, Sp1, and a labeled oligonucleotide containing the +55/+101 sequence of APP. As illustrated in the *top panel* of Fig. 7, both Sp1 (lane 6) and the TR/RXR heterodimer (lane 5) bound to this region of the gene. In contrast, in agreement with our previous description (5), no detectable complexes were observed when TR (lane 3) or RXR (lane 4) was used separately. In addition, as observed in the *last lane* of the autoradiogram, when the probe was simultaneously exposed to the receptors and Sp1, only the heterodimer TR/RXR, but not Sp1, was able to bind the labeled oligonucleotide, very likely indicating that, as proposed, binding of TR/RXR precludes binding of Sp1 to this DNA fragment. The displacement of Sp1 from the APP DNA fragment by TR/RXR was further confirmed in additional experiments carried out with increasing concentration of the heterodimer. As illustrated in the panel B of Fig. 7, Sp1 binding was progressively inhibited by increasing concentrations of TR/RXR. Moreover, the effect was more evident in the presence of T<sub>3</sub>.

In addition, a reduction in the expression of Sp1 could contribute to the inhibition of APP transcriptional



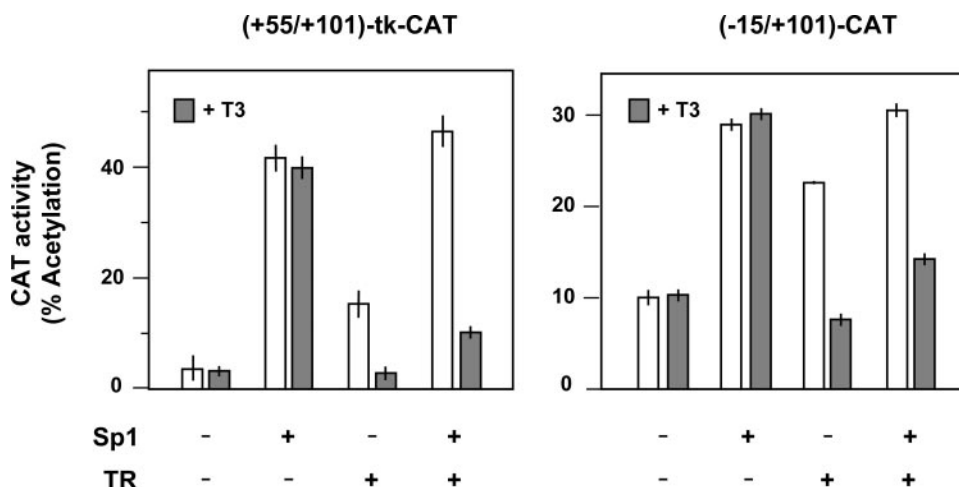
**Fig. 7.** Competition of Sp1 and TR/RXR for Binding to the APP Promoter

A, Gel mobility shift assays using the radiolabeled +55/+101 fragment of the APP gene, recombinant Sp1 (250 ng), and *in vitro* translated receptors (2  $\mu$ l), as indicated. The bands containing the TR/RXR heterodimer or Sp1 are identified by arrows. B, Assays were performed with Sp1 and increasing amounts (between 0.5 and 1.5  $\mu$ l) of the TR/RXR heterodimer in the presence and absence of 200 nM T<sub>3</sub>, as indicated. C, Sp1 levels were determined by Western blot in nuclear extracts from untreated N2a cells and cells treated for 24 and 48 h with 200 nM T<sub>3</sub>.

activity by the thyroid hormone. To exclude this possibility, we also analyzed the effect of T<sub>3</sub> on the nuclear levels of Sp1. As shown in panel C of Fig. 7, treatment of cells with T<sub>3</sub> by 24 and 48 h did not reduce Sp1 levels in N2a cells.

#### Regulation of APP Promoter Activity by Sp1 and TR

To analyze at the functional level the implication of this competition in the regulation of APP gene expression, N2a cells were cotransfected with vectors expressing TR, Sp1, or both, and CAT reporter plasmids containing the -15/+101 and +55/+101 fragments of the APP gene. Cells were incubated in the presence or in the absence of T<sub>3</sub> for 48 h, and CAT activity was measured as an indicator of the APP promoter activity. The results obtained are illustrated in Fig. 8. In the



**Fig. 8.** Regulation of the APP Promoter Activity by Sp1 and TR

CAT activity in N2a cells cotransfected with reporter plasmids that contain fragments +55/+101 or -15/+101 of the APP gene, and TR- and/or Sp1-expressing vectors as indicated in the figure. CAT was determined after a 48-h period of incubation in the presence or absence of 200 nM T<sub>3</sub>. Data are expressed as mean  $\pm$  SD of CAT activities obtained from three independent experiments.

absence of T<sub>3</sub>, CAT activity was significantly increased by both Sp1 and TR, the activity being maximal in cells expressing Sp1, and was not further increased by TR expression. In addition, T<sub>3</sub> did not affect either basal or Sp1-induced expression in the absence of TR, but effectively reversed the basal or Sp1-induced activity in TR-expressing cells.

## DISCUSSION

Binding to nTREs (17–21), interaction with other positive transcription factors (22), or competition for limiting transcriptional cofactors (23, 24) have been described to mediate negative regulation of gene expression by T<sub>3</sub>. In this report we have analyzed the regulation of APP gene expression by TR. We have previously described (5) that the unliganded receptor stimulates APP promoter activity and that T<sub>3</sub> significantly reverses this activation. Now, we have found that whereas the unliganded receptor can stimulate promoter activity without binding to DNA, the inhibitory effect of T<sub>3</sub> indeed requires binding of TR to sequences that, as occurs with other negatively regulated genes (6–10), are located downstream of the transcriptional start site. This region contains a TRE between positions +81 and +96 that preferentially binds heterodimers of TR with RXR. This is in contrast to observations with other nTREs (6, 7, 25), to which TRs bind as monomers or homodimers. Both effects, the T<sub>3</sub>-independent activation and the T<sub>3</sub>-induced repression of APP, appear to be isoform independent, because they are equally mediated by TR $\alpha$  or TR $\beta$  isoforms.

In contrast with the observed reduction of APP promoter activity in N2a cells transiently transfected with

TRs, T<sub>3</sub> is unable to regulate APP in the pituitary tumor cell line GH4C1 that expresses TRs (our unpublished observations), thus suggesting a cell-specific effect that likely requires the presence of factor/s that are not expressed in nonneuronal cells. However, T<sub>3</sub>-dependent repression of APP gene expression is not secondary to overexpression of TR in the neuroblastoma cells, because the inhibitory effect was also demonstrable in N2a $\beta$  cells that express physiological amounts of TR, with a T<sub>3</sub> nuclear binding capacity of 1.68 pmol/mg DNA (26). These levels are similar to the endogenous TR levels present in pituitary tumor cell lines (27). Moreover, the finding that the APP protein content is increased in some brain areas in hypothyroid animals (our unpublished observations) further confirm that TR could play a physiological role on APP gene expression in this tissue.

Our results also show that the presence of the TRE in the first exon of the APP gene is not sufficient to mediate the TR response, because the hormone-dependent and -independent effects were observed only in the presence of the TRE 5'-flanking sequences. The finding that the TRE by itself does not act as a functional response element suggests that competition of TR with other transcription factors, which recognize those sequences, could be involved in the observed regulation. The APP promoter region, located between nucleotides +55 and +101, contains a potential CRE and three Sp1 binding sites, preceding the TRE located at position +81/+96. However, whereas the Sp1 binding sites appear to be functional, the CRE-like sequence was unable to bind CREB, and repression by T<sub>3</sub> was yet observed when the CRE was partially removed from the responsive promoter sequences, which suggests that T<sub>3</sub>-dependent regulation of the APP promoter is CRE independent. In sup-

port of this conclusion we have also found that stimulation of CREB activity, by treatment with forskolin or overexpression of protein kinase A, was unable to stimulate APP promoter activity.

In contrast, our results are compatible with a mechanism in which TR competes with Sp1 for binding to DNA. This mechanism would have a particular relevance in the APP gene, which lacks the TATA box and which is, therefore, largely dependent on the constitutive activation induced by basal factors such as Sp1 (28). We have shown that expression of Sp1 stimulates APP promoter activity and that Sp1 binds to the +55/+101 region of the APP gene in EMSAs. However, the presence of the TR-RXR heterodimer precludes binding of Sp1 to this region of the APP gene. A similar mechanism involving overlapping DNA-binding sites has been already proposed for  $T_3$  suppression of Sp1-dependent transcription of the epidermal growth factor receptor promoter (29), for the regulation of the lactoferrin gene by estrogen, retinoic acid, and chicken ovalbumin upstream promoter receptors (30, 31), and for the regulation of the chicken lysozyme gene by TR and CCCTC-binding factor (CTCF) (32). The negative effect of  $T_3$  on the APP promoter could be also secondary to repression of Sp1 expression. However,  $T_3$  does not reduce Sp1 content in N2a $\beta$  cells, and as shown in Fig. 8, the inhibitory effect of  $T_3$  is also observed in cells transfected with TR and an expression vector for Sp1, thus indicating that inhibition of APP promoter activity is independent of the effects of  $T_3$  on Sp1 levels.

In addition to RXR/TR and Sp1, other proteins that form slow migrating complexes on the +55/+101  $T_3$ -responsive region of the APP gene are present in nuclear extracts of neuroblastoma cells. Characterization of these proteins is currently underway and will be important to analyze their possible contribution to the observed regulation. Preliminary results, obtained using mass spectroscopy, indicate that many of these proteins possess sequence homology with different members of the heterogeneous nuclear ribonucleoprotein family, a group of proteins that, according to previous descriptions, could play a role as transcriptional regulators (33, 34).

Whereas binding of TR to DNA has been reported to be essential for negative regulation of the TRH or TSH $\beta$  genes by  $T_3$  (12, 35), a mechanism based on protein-protein interactions, as opposed to TR binding directly to DNA sequences, has been proposed for negative regulation of the TSH $\alpha$  gene (13, 14). We have examined whether TR-mediated regulation of APP requires direct binding of TR to promoter sequences. Using a TR mutant that cannot bind DNA, we have found that the stimulatory effect induced by the unliganded receptor does not require binding of TR to the APP promoter. In contrast, and compatible with the competition hypothesis suggested above, the inhibitory effect of  $T_3$  was observed only in cells expressing the wild-type TR, which binds DNA. These results suggest a mechanism in which occupancy of

TR by  $T_3$  would promote binding of the receptor to the APP promoter. This model would be incompatible with a high-affinity binding of the unliganded receptor to the promoter and, accordingly, we have proved that a constitutively active receptor, obtained by fusion of full-length wild-type TR $\beta$  to the transcriptional activation domain of VP16 (13), is unable to activate CAT reporter genes containing the +55/+101 or -15/+101 fragments of the APP gene. These results strongly support the existence of a low-affinity-interacting site that *in vivo* likely binds TR only after  $T_3$  binding. In this respect, we have already indicated that TR binds to the APP promoter as a heterodimer with RXR. Because heterodimerization with RXR augments the DNA binding affinity of TR, and binding of  $T_3$  augments the interaction between TR and RXR (36, 37),  $T_3$  could repress transcription by increasing the affinity of TR-RXR heterodimers for DNA, which in turn would decrease the accessibility of other activating transcription factors (such as Sp1 or other still unidentified factors), likely involved in the constitutive stimulation of the APP promoter. In agreement with this hypothesis, we have also observed that stimulation by unliganded TR, which remains mainly unbound in the absence of ligand, is also reversed when its binding to the APP promoter is facilitated by coexpression of RXR (data not shown).

In addition, in the case of negative regulation of the TSH $\alpha$  gene by  $T_3$ , it has been suggested that unliganded TR recruits CoRs and withdraws HDACs from the basal promoter causing activation, whereas  $T_3$  binding dissociates the CoRs/HDACs and recruits coactivators to restrict access to histone acetylases to components of the basal promoter, thereby causing ligand-dependent repression. Moreover, the stimulation induced by the unliganded receptor would involve competition for, and squelching of, a limiting amount of CoRs that are bound to other factors on the promoter (13, 14). We have observed that overexpression of CoRs or HDACs reverses activation of the APP promoter by unliganded TR (data not shown). These findings strongly suggest a role for CoRs/HDAC in TR-mediated regulation of APP. However, the mechanisms that mediate the effects of TR on the APP gene appear to be essentially different from those described for other negatively regulated genes, in which expression of silencing mediator of retinoid and thyroid hormone receptor or nuclear receptor corepressor markedly enhances activation by unoccupied TR (14). If withdrawal of CoRs is involved in stimulation by the unliganded TR, it is tempting to speculate that this receptor in the absence of  $T_3$  could have a more general activator role in other housekeeping genes in which transcriptional activation is largely dependent on Sp1, particularly those having TR and Sp1 binding sites.

On the other hand, it has been also proposed that DNA binding of TR would be not necessary in TR-mediated control of negatively regulated genes (14). Using a TR mutant we have demonstrated that in the



absence of  $T_3$  the stimulation of basal promoter activity does not require a TR bound to DNA, a circumstance that likely contributes to the APP promoter stimulation by a double mechanism: 1) making possible binding of other transcription factors to DNA; and 2) by squelching of CoRs/HDAC that finally causes activation. In contrast, the  $T_3$ -induced repression requires binding of TR to DNA and likely involves displacement from the DNA of other general transcription factors that also bind to that region of the APP promoter. However, because to be functional the proposed mechanism should allow the interchange of those factors from DNA, it would be incompatible with a high-affinity TR binding site, and these results strongly support the existence of a low-affinity-interacting site that likely binds TR only under optimal conditions.

In summary, our results suggest a dual mechanism in which unliganded TR does not interact with DNA, allowing binding of Sp1 and the subsequent transcriptional activation by this factor. In addition, unliganded TR might also contribute to promoter activation by withdrawal of CoRs and HDACs from the promoter. Binding of  $T_3$  would facilitate formation of TR-RXR heterodimers, which would then bind to the TRE and displace Sp1 from the overlapping sequences +64/+83, blocking activation by this transcription factor. Why TR/RXR complex fails to activate transcription once bound to the APP element after  $T_3$  addition is still unknown. However, again, displacement of strong activators such as Sp1 factors could be involved. This mechanism could be particularly relevant in the case of the APP gene, because Sp1 is required for basal transcription of genes that, like the APP gene, lack a TATA box (28). The equilibrium between binding of two transcription factors, TR and Sp1, to overlapping sequences of DNA would allow a precise control of the APP gene expression. Because binding of  $T_3$  to its receptors fits a sigmoidal kinetics (38, 39), small increases of  $T_3$  concentration would be sufficient to significantly enhance TR occupancy, which in turn would facilitate binding of the TR/RXR heterodimer to the low-affinity binding site of the APP gene, and to cause the displacement of Sp1 and the consequent reduction of promoter activity. Clearly, additional work will be necessary to clarify the mechanisms of the functional interaction between TR and Sp1 and its role in regulation of gene expression.

## MATERIALS AND METHODS

### Cell Culture

Murine N2a neuroblastoma cells were grown in DMEM supplemented with 10% fetal bovine serum as previously described by Ortiz-Caro *et al.* (39). Previous to the experiments, the culture medium was replaced with a similar medium containing serum depleted of thyroid hormone by treatment with resin AG1X8 as described by Samuels *et al.* (40), and the cells were then incubated in this medium for an additional

24-h period before the beginning of the experiments. N2a- $\beta$  cells, a subclone that constitutively expresses the  $\beta$ -isoform of TR (TR $\beta$ ), were obtained from Dr. Dussault's laboratory. N2a- $\beta$  cells were grown as previously described by Lebel *et al.* (26), and the experiments were carried out in the same medium and conditions described for N2a cells.

### Reporter Plasmids and Expression Vectors

The CAT reporter plasmid containing the –1099/+106 fragment of the human APP gene has been previously described (41). 5'-Deletions to –15, +55, or +75 bp were prepared by PCR from the original –1099/+105 bp fragment, kindly provided by Dr. Lahiri's laboratory, and subcloned into the *Bam*H1 site of pBL-CAT8. The reporter constructs +55/+101-, +63/+101-, +75/+101-, or +55/+79-CAT consist of a single copy of those APP fragments inserted in front of a tk promoter driving the expression of the CAT gene. Mutant M1 contains the mutations of the upstream hemisite of the TRE indicated in *boldface* (CAACTAGACGAAGGACG). In M2, mutations are present in the intervening nucleotides of the response element (CGGGCAGACTCAGGACG), and M3 is mutated in the downstream hemisite (CGGGCAGACGAAACTG), also affecting the downstream Sp1 binding site. These mutations were introduced in the +55/+101 fragment ligated to the tk promoter. The cDNAs encoding Sp1, TR $\alpha$ , and RXR $\alpha$ , as well as the TR mutant C51G, are inserted into the *Eco*I site of the expression vector pSG5, which contains the simian virus 40 early promoter. The construct containing full-length TR coding sequences fused to the activation domain of VP16 was a kind gift of Dr. J. L. Jameson, and it has been described previously (13).

### DNA Transfection

N2a cells cultured in DMEM containing 10% of thyroid hormone-depleted fetal calf serum were transfected by the calcium phosphate coprecipitation method with 300 ng of reporter plasmids. A luciferase reference vector (100 ng) was simultaneously used as an internal control of the transfection efficiency. In cotransfection experiments, 300 ng of reporter plasmid and 1  $\mu$ g of the corresponding receptor expression vector were used. After 16 h of incubation in the presence of calcium phosphate, the medium was discarded and washed with 5 ml PBS. A new medium containing 0.5% thyroid hormone-depleted serum was added, and the cells were then incubated for an additional period of 48 h in the presence or absence of 200 nM  $T_3$ . Each treatment was performed in duplicate cultures that normally showed less than 5–15% variation in CAT activity, which was determined by incubation of [ $^{14}$ C]chloramphenicol with cell lysate protein. After autoradiography, the nonacetylated and acetylated [ $^{14}$ C]chloramphenicol was quantified, and the data are expressed as the mean  $\pm$  SD of the percent of acetylated forms after each treatment. Each experiment was repeated at least two to three times with similar relative differences in regulated expression.

### Mobility Shift Assays

Synthetic oligonucleotides containing the TR-binding sequences of the human APP promoter (+55/+101), or the consensus TREpal sequence, were end labeled with [ $\alpha$ - $^{32}$ P]dCTP by using the Klenow fragment of *Escherichia coli* DNA polymerase and then incubated with *in vitro* translated receptors, recombinant Sp1 (obtained from Promega Corp., Madison, WI), or with nuclear extracts obtained from N2a- $\beta$  cells. cDNAs for TR and RXR in pSG5 were transcribed and translated *in vitro* with the TNT kit (Promega) following the manufacturer's recommendations. The nuclear extracts were obtained by the method of Andrews and Faller (42). For gel

retardation assays, purified Sp1 (250 ng), translated receptors (between 0.5 and 2  $\mu$ l), or nuclear extracts (5  $\mu$ g) were incubated on ice for 15 min in a buffer (20 mM Tris HCl, pH 7.5; 75 mM KCl; 1 mM dithiothreitol; 5  $\mu$ g/ml BSA; 13% glycerol) containing 3  $\mu$ g poly (dI-dC) and then were incubated for 15–20 min at room temperature with approximately 70,000 cpm of the double-stranded labeled oligonucleotide. Unprogrammed reticulocyte lysate was used as a control for nonspecific binding. For competition experiments, increasing concentrations of unlabeled double-stranded oligonucleotide, or an oligonucleotide containing the consensus sequence TREpal (5'AGGTCATGACCT-3'), were added to the binding reaction mixture. DNA-protein complexes were resolved on 5% nondenaturing polyacrylamide gels containing 0.5% Tris-borate-EDTA buffer. The gels were dried and autoradiographed at –70 C.

### Western Blot Analysis

Extracts from N2a $\beta$  cells incubated in the presence or in the absence of T<sub>3</sub> were run in 10% acrylamide gels. The proteins were transferred to a nitrocellulose membrane and identified by chemiluminescence after incubation with a 1:2000 dilution of the specific antibody sc-59 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) raised against the transcription factor Sp1.

### Acknowledgments

We thank Dr. A. Aranda for critical reading of the manuscript and Ana Pascual for valuable linguistic help.

Received July 2, 2003. Accepted December 31, 2003.

Address all correspondence and requests for reprints to: Dr. A. Pascual, Instituto de Investigaciones Biomédicas (C.S.I.C.), Arturo Duperier 4, 28029 Madrid, Spain. E-mail: apascual@iib.uam.es.

This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (SAF2000-0177). A.V. was recipient of a fellowship from GlaxoSmithKline and Consejo Superior de Investigaciones Científicas. J.S. was supported by a fellowship from the Comunidad Autónoma de Madrid.

### REFERENCES

- Selkoe DJ 1994 Cell biology of the amyloid  $\beta$ -protein precursor and the mechanism of Alzheimer's disease. *Annu Rev Cell Biol* 10:373–403
- Mattson MP, Cheng B, Culwell AR, Esch FS, Lieberburg I, Rydel RE 1993 Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the  $\beta$ -amyloid precursor protein. *Neuron* 10:243–254
- Fukuchi K, Kamino K, Deeb SS, Smith AC, Dang T, Martin GM 1992 Overexpression of amyloid precursor protein alters its normal processing and is associated with neurotoxicity. *Biochem Biophys Res Commun* 182:165–173
- Latasa MJ, Belandia B, Pascual A 1998 Thyroid hormones regulate  $\beta$ -amyloid gene splicing and protein secretion in neuroblastoma cells. *Endocrinology* 139:2692–2698
- Belandia B, Latasa MJ, Villa A, Pascual A 1998 Thyroid hormone negatively regulates the transcriptional activity of the  $\beta$ -amyloid precursor protein gene. *J Biol Chem* 273:30366–30371
- Carr FE, Wong NC 1994 Characteristics of a negative thyroid hormone response element. *J Biol Chem* 269:4175–4179
- Hollenberg AN, Monden T, Flynn TR, Boers ME, Cohen O, Wondisford FE 1995 The human thyrotropin-releasing hormone gene is regulated by thyroid hormone through two distinct classes of negative thyroid hormone response elements. *Mol Endocrinol* 9:540–550
- Madison LD, Ahlquist JA, Rogers SD, Jameson JL 1993 Negative regulation of the glycoprotein hormone  $\alpha$  gene promoter by thyroid hormone: mutagenesis of a proximal receptor binding site preserves transcriptional repression. *Mol Cell Endocrinol* 94:129–136
- Wood WM, Kao MY, Gordon DF, Ridgway EC 1989 Thyroid hormone regulates the mouse thyrotropin  $\beta$ -subunit gene promoter in transfected primary thyrotropes. *J Biol Chem* 264:14840–14847
- Perez-Juste G, Aranda A 1999 Differentiation of neuroblastoma cells by phorbol esters and insulin-like growth factor 1 is associated with induction of retinoic acid receptor  $\beta$  gene expression. *Oncogene* 18:5393–5402
- Cohen RN, Putney A, Wondisford FE, Hollenberg AN 2000 The nuclear corepressors recognize distinct nuclear receptor complexes. *Mol Endocrinol* 14:900–914
- Shibusawa N, Hollenberg AN, Wondisford FE 2003 Thyroid hormone receptor DNA binding is required for both positive and negative gene regulation. *J Biol Chem* 278:732–738
- Tagami T, Park Y, Jameson JL 1999 Mechanisms that mediate negative regulation of the thyroid-stimulating hormone  $\alpha$  gene by the thyroid hormone receptor. *J Biol Chem* 274:22345–22353
- Tagami T, Madison LD, Nagaya T, Jameson JL 1997 Nuclear receptor corepressors activate rather than suppress basal transcription of genes that are negatively regulated by thyroid hormone. *Mol Cell Biol* 17:2642–2648
- Karin M 1998 New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? *Cell* 93:487–490
- Suske G 1999 The Sp-family of transcription factors. *Gene* 238:291–300
- Chatterjee VK, Lee JK, Rentoumis A, Jameson JL 1989 Negative regulation of the thyroid-stimulating hormone  $\alpha$  gene by thyroid hormone: receptor interaction adjacent to the TATA box. *Proc Natl Acad Sci USA* 86:9114–9118
- Carr FE, Kaseem LL, Wong NC 1992 Thyroid hormone inhibits thyrotropin gene expression via a position-independent negative L-triiodothyronine-responsive element. *J Biol Chem* 267:18689–18694
- Pombo PM, Barettino D, Espliguero G, Metsis M, Iglesias T, Rodríguez-Pena A 2000 Transcriptional repression of neurotrophin receptor trkB by thyroid hormone in the developing rat brain. *J Biol Chem* 275:37510–37517
- Perez-Juste G, Garcia-Silva S, Aranda A 2000 An element in the region responsible for premature termination of transcription mediates repression of c-myc gene expression by thyroid hormone in neuroblastoma cells. *J Biol Chem* 275:1307–1314
- Satoh T, Yamada M, Iwasaki T, Mori M 1996 Negative regulation of the gene for the preprothyrotropin-releasing hormone from the mouse by thyroid hormone requires additional factors in conjunction with thyroid hormone receptors. *J Biol Chem* 271:27919–27926
- Zhu YS, Yen PM, Chin WW, Pfaff DW 1996 Estrogen and thyroid hormone interaction on regulation of gene expression. *Proc Natl Acad Sci USA* 93:12587–12592
- Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Glass B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG 1996 A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85:403–414
- Zhu XG, Park KS, Kaneshige M, Bhat MK, Zhu Q, Mariash CN, McPhie P, Cheng SY 2000 The orphan nuclear receptor Ear-2 is a negative coregulator for thyroid hor-

- negative nuclear receptor function. *Mol Cell Biol* 20: 2604–2618
25. Hallenbeck PL, Phyllaier M, Nikodem VM 1993 Divergent effects of 9-cis-retinoic acid receptor on positive and negative thyroid hormone receptor-dependent gene expression. *J Biol Chem* 268:3825–3828
26. Lebel JM, Dussault JH, Puymirat J 1994 Overexpression of the  $\beta$  1 thyroid receptor induces differentiation in neuro-2a cells. *Proc Natl Acad Sci USA* 91:2644–2648
27. Samuels HH, Stanley F, Shapiro LE 1976 Dose-dependent depletion of nuclear receptors by L-triiodothyronine: evidence for a role in induction of growth hormone synthesis in cultured GH1 cells. *Proc Natl Acad Sci USA* 73:3877–3881
28. Pugh BF, Tjian R 1990 Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* 61: 1187–1197
29. Xu J, Thompson KL, Shephard LB, Hudson LG, Gill GN 1993 T3 receptor suppression of Sp1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites. *J Biol Chem* 268:16065–16073
30. Lee MO, Liu Y, Zhang XK 1995 A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactoferrin gene. *Mol Cell Biol* 15:4194–4207
31. Liu Y, Teng CT 1992 Estrogen response module of the mouse lactoferrin gene contains overlapping chicken ovalbumin upstream promoter transcription factor and estrogen receptor-binding elements. *Mol Endocrinol* 6:355–364
32. Lutz M, Burke LJ, LeFevre P, Myers FA, Thorne AW, Crane-Robinson C, Bonifer C, Filippova GN, Lobanenko V, Renkawitz R 2003 Thyroid hormone-regulated enhancer blocking: cooperation of CTCF and thyroid hormone receptor. *EMBO J* 22:1579–1587
33. Chen H, Hu B, Allegretto EA, Adams JS 2000 The vitamin D response element-binding protein. A novel dominant-negative regulator of vitamin D-directed transactivation. *J Biol Chem* 275:35557–35564
34. Kim MK, Nikodem VM 1999 hnRNP U inhibits carboxy-terminal domain phosphorylation by TFIIH and represses RNA polymerase II elongation. *Mol Cell Biol* 19:6833–6844
35. Bodenner DL, Mroczynski MA, Weintraub BD, Radovick S, Wondisford FE 1991 A detailed functional and structural analysis of a major thyroid hormone inhibitory element in the human thyrotropin  $\beta$ -subunit gene. *J Biol Chem* 266:21666–21673
36. Collingwood TN, Butler A, Tone Y, Clifton-Bligh RJ, Parker MG, Chatterjee VK 1997 Thyroid hormone-mediated enhancement of heterodimer formation between thyroid hormone receptor  $\beta$  and retinoid X receptor. *J Biol Chem* 272:13060–13065
37. Zhang J, Zamir I, Lazar MA 1997 Differential recognition of liganded and unliganded thyroid hormone receptor by retinoid X receptor regulates transcriptional repression. *Mol Cell Biol* 17:6887–6897
38. Ortiz-Caro J, Montiel F, Pascual A, Aranda A 1986 Modulation of thyroid hormone nuclear receptors by short-chain fatty acids in glial C6 cells. Role of histone acetylation. *J Biol Chem* 261:13997–14004
39. Ortiz-Caro J, Yusta B, Montiel F, Villa A, Aranda A, Pascual A 1986 Identification and characterization of L-triiodothyronine receptors in cells of glial and neuronal origin. *Endocrinology* 119:2163–2167
40. Samuels HH, Stanley F, Casanova J 1979 Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology* 105:80–85
41. Lahiri DK, Robakis NK 1991 The promoter activity of the gene encoding Alzheimer  $\beta$ -amyloid precursor protein (APP) is regulated by two blocks of upstream sequences. *Brain Res Mol Brain Res* 9:253–257
42. Andrews NC, Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 19:2499



**Molecular Endocrinology** is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.